From food to pharmaceuticals: the evolution of nitrosamine analysis

By Melroy Feit

N-nitrosamines are one of the most toxic and predictably carcinogenic families of organic compounds encountered in the environment. These compounds may be found in food products, whiskey, agricultural chemicals, cosmetics, and rubber products. In addition, nitrosamines may be formed within the body as a result of a reaction between amine containing drugs and nitrites. Although no specific cases of human cancer have been directly related to exposure to nitrosamines, these compounds have been found to cause cancer in laboratory animals.

All animal species tested over the past 40 years have been susceptible to the carcinogenic effects of N-nitrosamines, and nearly all N-nitrosamines can cause cancer in at least one animal species. In fact, with suitable combinations of N-nitrosamines and test animals, cancer can be induced in almost any organ in the body. There are more than 300 known nitrosamines in foods and over 90% of these are known to be carcinogens, mutagens, or teratogens.

Although N-nitrosamines can produce cancer at very low doses in animals, information is incomplete on the risk of tumor induction in humans at normal exposure levels. The potential of nitrosamines to cause cancer in humans poses unique issues because these compounds can be formed from common chemicals under conditions routinely encountered in manufacturing, processing, and storage of chemical products. As a result, there has been increased interest in the possible migration of nitrosamines from rubber components used in pharmaceutical packaging or delivery systems into pharmaceutical products. This has resulted in increased testing of this potential source of exposure.

Sources of Exposure

Although human exposure to nitrosamines can occur through a variety of routes, food has traditionally been looked at as the primary source. In humans, nitrosamines may be formed in the stomach when free nitrites are ingested and react with amines from foods or the body. Although there have been concerns about the use of nitrite

Figure 1: Significant Milestones in Nitrosamine Analysis

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Table 1. Estimated Exposure to Nitrosamines in the United States (ug/day)

in the processing of cured meat products, only about 2% of the average daily intake can be attributed to this source. Research on cured meats has shown that nitrosamines (i.e., N-nitrosodimethylamine, N-nitrosopiperidine, N-nitrosopyrolidine, N-nitrosothiazolidine) are only found in bacon after it has been fried. Although elastic rubber netting on cured meats is a potential source of nitrosamines, studies have uncovered no significant differences in nitrosamines detected in cured cooked hams with netting and in control hams.

In 1978, the United States Department of Agriculture eliminated the use of nitrate in most products. Nitrite use is limited and must be accompanied by a reducing agent (e.g., ascorbic acid, alpha-tocopherol) which prevents the formation of nitrosamines. The Food and Drug Administration (FDA) has also established action levels of 5 parts per billion (ppb) of NDMA in malt beverages and 10 ppb in malt barley.

However, exposure to nitrosamines is not limited to food and beverages. Because the precursors of nitrosamines are widely distributed in the environment, exposure can occur from a number of sources, including soil, water, and even the interiors of new cars.

Nitrosamine contamination of commercial products can occur when nitrosamines are generated as by-products of chemical manufacturing processes. This is of increased concern when the process includes a nitration, as trace quantities of nitrite react with nitrogen functionalities such as amines or amides present in the molecule to form N-nitrosamines. Other potential sources of contamination include starting materials, reagents, or water used in formulation, and nitrosation during storage. Because functional groups containing nitrogen are frequent compo-

For example, the action of nitrite on nitrogen functionalities in a pesticide in storage can generate N-nitrosamines. The nitrosamines occur either as N-nitrosated forms of the base structure or as N-nitrosamines of amines such as diethylamine and diethanolamine used to make salts of the parent pesticide. Workers in the production of rubber and related items have traditionally been at high risk for exposure to nitrosamines. Although the maximum exposure is during the curing phase, workers at all stages of the process are subject to some exposure. Monitoring of worker exposure can be accomplished using a cartridge, which absorbs the nitrosamines in the air. The levels of nitrosamines are determined by extracting the compounds from the cartridge and measuring them using chromatographic techniques.

A variety of personal care items such as cosmetics, lotions, and shampoos have been shown to contain N-nitrosodiethanolamine and other N-nitrosoalkanolamines. The presence of these compounds is a result of reactions with the parent alkanolamines, which are important constituents in these products. Ranges have been found to be from less than 20 ppb to 150 parts per million (ppm). Various formulations of drugs containing aminopyramine, disulfiram, or oxytetracycline have also been found to contain different levels of nitrosamines.

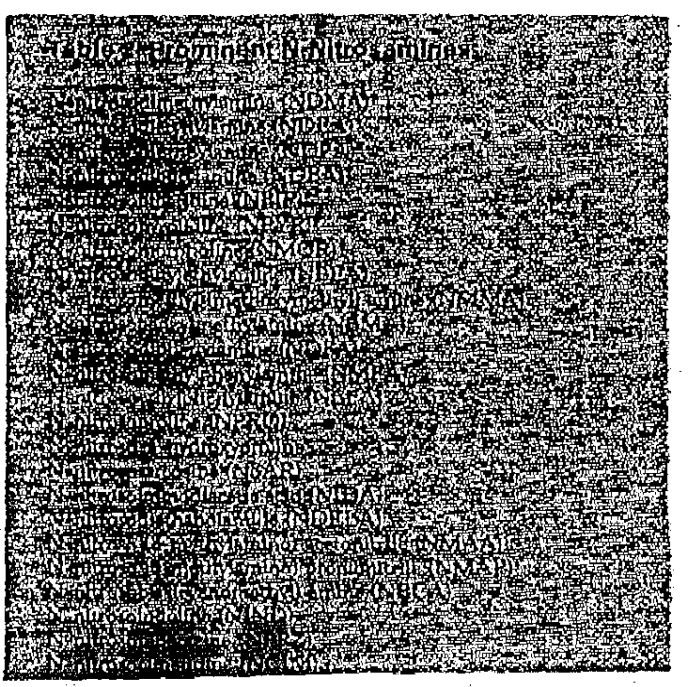
Table 2. Prominent Nitrosamines

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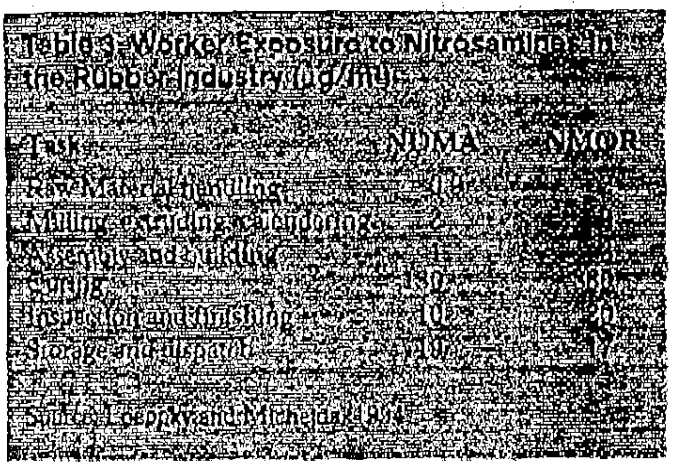

Table 3. Worker Exposure to Nitrosamines in the Rubber Industry (µg/m³)

Task	NDMA	NMOR
Raw Material handling	[illegible]	[illegible]
Milling, extruding, calendering	[illegible]	[illegible]
Assembly and building	[illegible]	[illegible]
Curing	130	360
Inspection and finishing	10	20
Storage and dispatch	10	17

Source: Loeppky and Michejda 1994

In 1981, research showed the occurrence of volatile N-nitrosamines (i.e., NDMA, NDEA, NDBA, and NPIP) in rubber baby bottle nipples at levels up to 1,000 ppb. This was due to the use of dialkylamine-derived accelerators and stabilizers in the production of the rubber. A modification in the manufacturing process led to a reduction in levels to below 10 ppb which meets FDA limits established in 1985 of 10 ppb for any individual N-nitrosamine. This 10 ppb level is determined by measuring the amounts in 3 aliquots from a composite matrix of 12 nipples. The issue of nitrosamine migration from a rubber product has other implications, including those associated with pharmaceutical containers.

Pharmaceutical Containers

In 1997, the FDA published a guidance document for comment purposes entitled "Guidance for Industry: Submission of Documentation in Drug Applications for Container Closure Systems Used for the Packaging of Human Drug and Biologics." This document addresses the general principles associated with submitting information on packaging materials for human drugs and biologics. In addition to addressing issues such as suitability, safety is also a primary concern. The Federal Food, Drug, and Cosmetic Act (FFDCA) specifies that a drug will be deemed to be adulterated if its container is composed, in whole or in part, of any poisonous or deleterious substance which may render the contents injurious to health. Because nitrosamines are commonly present in rubber and elastomeric products, any packaging component made of these substances is a potential source of exposure.

Several individual components (e.g., vials, liners, closures, stopper overseals, administration ports) comprise the container closure (i.e., packaging) system. Primary packaging components are those that will be in direct contact with the dosage form. Secondary components are those which do not come in contact with the dosage. It is the primary components, of course, which are of principle interest when determining potential sources of contamination. The guidance document published for comment by the FDA identifies information that must be submitted for all drug products, as well as specific data of interest with a particular dosage type.

Inhalation drug products are of special concern due to the nature of the packaging components. The metered-dose inhaler is one of the primary systems for delivery of medication for asthma and bronchial conditions. Key components (i.e., gaskets, seals, "o"-rings) of these inhalers are manufactured from elastomeric substances and testing is now conducted to determine the levels of nitrosamines present in the components themselves, as well as levels in the drug after being expelled from the inhaler. The chemical nature of these compounds has a significant effect on the testing methodology used.

Chemistry and Classification

N-nitroso compounds have an N-NO bond as their key structural feature and may be divided into two broad categories: nitrosamines and nitrosamides. Compounds in the nitrosamide class have a carbonyl group attached to the nitrogen bearing the NO group. These compounds are known to be direct mutagens and include the reactive N-nitrosamides, N-nitrosocarbamates, and N-nitrosoureas. In nitrosamines, the N-NO group is attached to an alkyl or aryl group. Unlike nitrosamides, nitrosamines must be metabolized in order to elicit mutagenic or carcinogenic properties.

Nitrosamines can be formed under acidic, basic, or neutral conditions by the reaction of primary, secondary, and tertiary amines as well as quaternary ammonium salts, with nitrite ions or a variety of other nitrosating agents (e.g., oxides of nitrogen). Some research has shown that the chemical structure of the N-nitrosamines affects their ability to induce cancer. For example, the chain length of symmetric dialkylnitrosamines is inversely related to the carcinogenic potency of this group. In cyclic nitrosamines, the cancer causing activity is directly related to ring size.

Nitrosamines can be synthesized by reacting (e.g., heating) a secondary amine in the presence of sodium nitrite and an acid such as acetic acid. Nitrous acid is generated *in situ*. The mechanism is thought to involve intermediate formation of N_2O_3, which is the active nitrosating reagent. When primary amines are treated with nitrous acid, they form diazonium salts instead of N-nitrosamines. Tertiary

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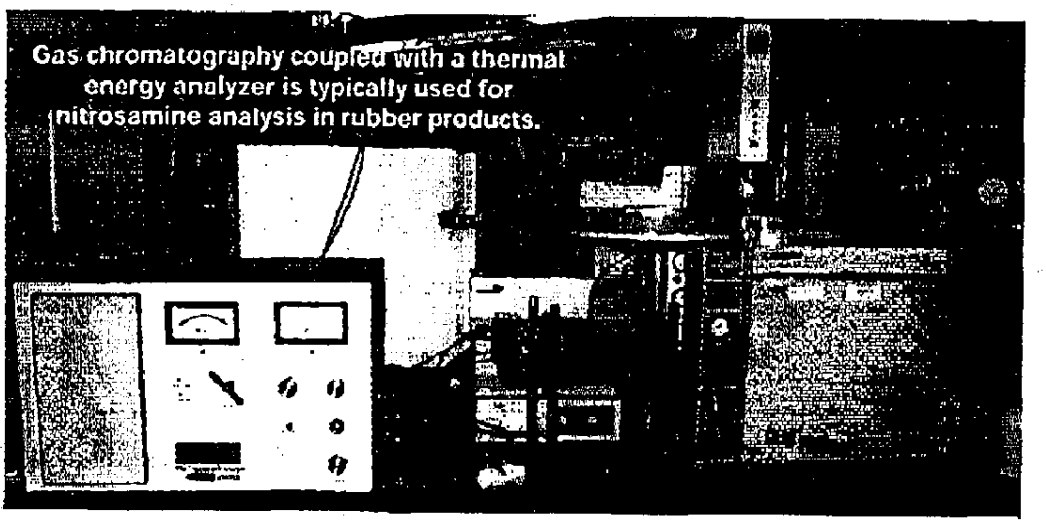
amines can yield N-nitrosamines, although one of the groups attached to the amine is cleaved and the product is a nitroso derivative of a secondary amine.



Gas chromatography coupled with a thermal energy analyzer is typically used for nitrosamine analysis in rubber products.

The N–NO bond in N-nitrosamines is relatively weak, and instability is often caused as a result of cleavage of this bond. N-nitrosamines are extremely sensitive to ultra-violet (UV) light which, in a reaction similar to thermolysis, will break the weak N–NO bond. Thermolysis of N-nitrosamines results in homolysis of the N–NO bond, forming an amine radical and a nitrosyl radical. In analysis, this reaction has been used as the basis for a selection of a detector for N-nitrosamines as well as a confirmatory test. While most nitrosamines require high-intensity UV irradiation to show significant decomposition, especially sensitive nitrosamines like N-nitrosoatrazine are readily decomposed under fluorescent lights, and must be handled in the laboratory under yellow or red lights.

N-nitrosamines vary widely in their physical and chemical properties, and these variations lead to differences in the cleanup and instrumental techniques used for their determination. These differences have resulted in the division of nitrosamines into two broad classes. N-nitrosamines can be categorized as either polar or nonpolar based on their solubility properties. Nonpolar nitrosamines are typically isolated from a matrix by extraction into nonpolar organic solvents such as dichloromethane.

In contrast, polar nitrosamines may be only marginally soluble in nonpolar organic solvents. As a result, solvents such as lower alcohols, acetone, and ethyl acetate are used for extraction of these compounds. Another classification system for N-nitrosamines is based upon the distillation techniques used for separation of N-nitrosamines from the matrix. Those that distill readily have been termed volatile N-nitrosamines. Generally, these parallel the nonpolar nitrosamine classification. Nonvolatile nitrosamines are usually synonymous with the polar N-nitrosamines.

Analytical Methodology

Methods to determine the presence of nitrosamines involve extraction and concentration of the sample, followed by chromatographic analysis. Samples being analyzed for nitrosamines vary significantly in their chemical properties (i.e., aqueous-based emulsions, organic solids, and mixed salts). As a result, unique sample preparation, purification, and concentration techniques must often be developed that will allow analysis of trace quantities of nitrosamines.

N-nitrosamines behave well when chromatographed under conditions typically used in both gas chromatography (GC) and high-pressure liquid chromatography (HPLC). In both separation techniques, the thermal energy analyzer (TEA) is the detector of choice for identification and analysis of the compounds.

Although levels of specific N-nitrosamines may be measured using the standard detectors used in chromatography (e.g., flame ionization, electron conductivity, UV, nitrogen-phosphorus), they offer no advantages for N-nitrosamines over other compounds present in the matrix in terms of increased sensitivity or selectivity and interferences often result. The thermal energy analyzer (TEA) was developed in the 1970s to selectively detect N-nitroso compounds. The detection principle is based on the thermal cleavage reaction of N-nitrosamines and the chemiluminescence of excited-state nitrogen dioxide produced by oxidation of the initially generated nitrosyl radical. The components of a TEA detector are:

- Thermolysis chamber heated to 500°C or more
- Mechanism for trapping solvents and species less volatile than the nitrosyl radical
- Oxidizing chamber with an ozone generator
- Photochemical cell to detect the emission of light generated by the nitrogen dioxide returning to ground level.

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For better separation and analysis, the TEA can be interfaced to a GC or HPLC. The GC interface is best accomplished by connection of the GC outlet to the TEA pyrolyzer. The gases that comprise the bulk of the material introduced into the TEA from the GC can pass through the TEA without an effect on the analysis. As a result, little treatment of the introduced stream is necessary, and the detector produces a very clean signal with good sensitivity.

Interfacing an HPLC to the TEA requires a mechanism for removing the HPLC mobile phase from the inlet stream before ionization and detection. This is accomplished by passing the stream through a series of cold traps after the pyrolyzer. These traps capture the solvent but allow the nitrosyl radical to flow through unimpeded. Due to the buildup of solvent in the trapping system, the TEA signal loses some sensitivity compared to GC/TEA, but still produces acceptable response with selective detection of N-nitrosamines. The HPLC method is generally used for polar nitrosamines, while GC interfaced with TEA is used for nonpolar.

Gas chromatography has been performed using both packed and capillary columns. Because volatilization is a prerequisite for successful GC analysis, polar, nonvolatile nitrosamines such as NDELA cannot be analyzed. However, derivatization increases volatility, and permits GC analysis. For example, NDELA is cleanly derivatized by N, O- bis (trimethylsilyl) trifluoroacetamide to generate a product that gives a sharp peak upon GC analysis.

The HPLC method offers some advantage for thermally sensitive amines because high temperatures are not required. Both reverse phase and normal phase chromatography can be used, although normal phase systems have advantages for the TEA detector.

Table 6. Sensitivity and Precision of Nitrosamine Analysis in Rubber Products

Compound	Limit of Detection (LOD) (ng/mL)	Limit of Quantitation (LOQ) (ng/mL)
NDMA	10	30
NDEA	10	30
NDBA	10	30
NDP	10	30
NDHA	10	30
NMOR	10	30

After conducting system suitability procedures, a nitrosamine standard solution is injected and analyzed. Using the same chromatographic conditions, the concentrated sample extract is injected and analyzed. Peak responses of the nitrosamines in the standard and sample are measured and the nitrosamine content is determined.

Dose Inhaler Analysis

Nitrosamine analysis for pharmaceutical products is done within cGMP guidelines. When analyzing rubber products such as the gaskets in metered-dose inhalers, volatile N-nitrosamines are extracted with methylene chloride. The solution is then made alkaline. Methylene chloride and water are distilled and any nitrosamines in the aqueous phase are partitioned back into the methylene chloride. The extract is concentrated for detection by GC/TEA. Standard solutions are also injected and detected for comparison and identification.

The procedure used to analyze the contents of a metered dose inhaler for nitrosamines involves expelling the contents of the inhaler into methylene chloride using a sampling needle. This extract is concentrated and analyzed using GC/TEA.

Table 6. Sensitivity and Precision of Nitrosamine Analysis in the Contents of Metered Dose Inhalers

Component	LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/Actuation)	LOQ (ng/Actuation)
NDMA	5	10	10	20
NDEA	5	10	10	30
NDBA	5	20	10	40
NDPA	5	15	10	30
NPIP	10	20	20	40
NMOR	10	20	20	40

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Dichloromethane is added to a graduated cylinder. A sampling needle is inserted in the canister (inhaler). In several phases, bursts of sample are then expelled into the liquid. The contents are expelled until the container is empty. The sampling needle is then removed from the canister and rinsed with dichloromethane. The contents of the cylinder are concentrated with nitrogen, a desired volume is attained, and the samples transferred to a GC vial for analysis.

The elastomeric components of the inhalers have been found to contain 10 – 200 ng/mL of the individual nitrosamines of interest (i.e., NDMA, NDEA, NDBA, NMOR, NPIP, and NPYR). In general, the nitrosamine found most frequently, and in the highest concentration, is NDMA. Significantly lesser amounts of the other compounds were detected.

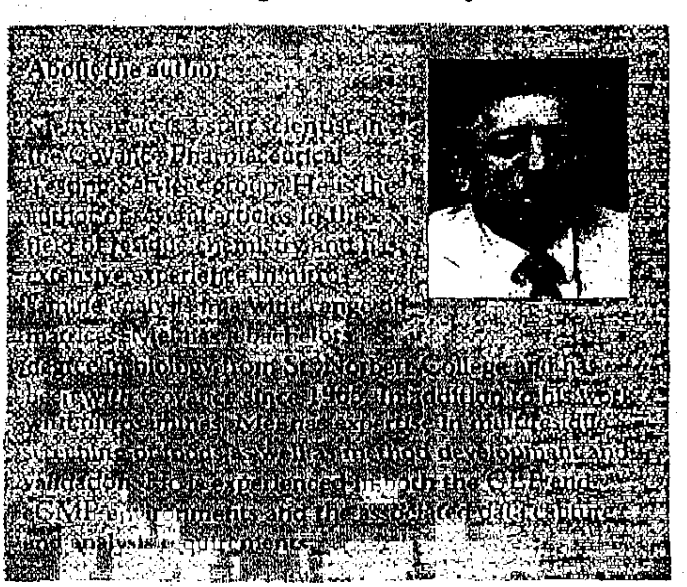
A possible contributor to increased nitrosamine levels in some samples is the diluent used in the inhalers. For example, inhalers which use certain fluorocarbon products as the propellant generally, have higher levels of nitrosamines than those that use other propellants.

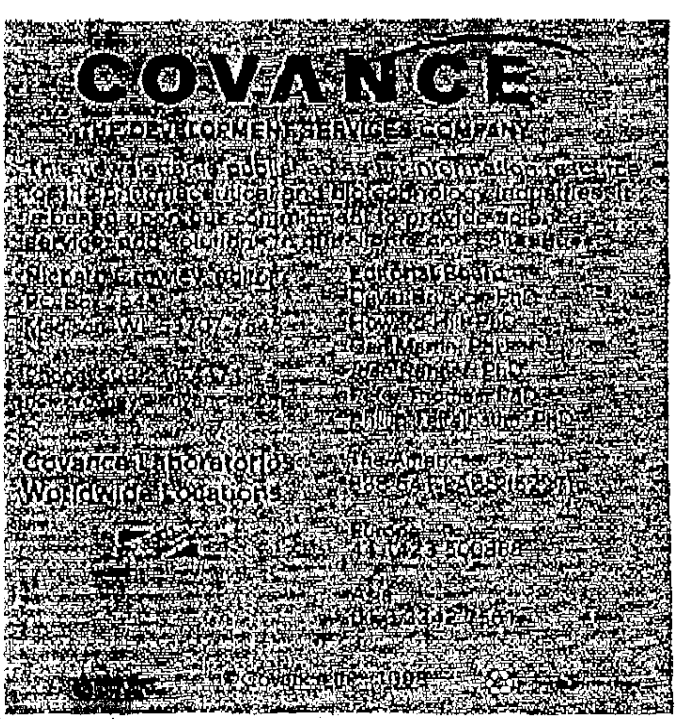
Advancing Interest and Methodology

The ability to prevent the potential harmful effects caused by nitrosamines depends on gaining knowledge of how these compounds are formed and implementing effective analytical methods for detecting them. As the amount of information concerning N-nitrosamines grows, other analytical methods for nitrosamine analysis have been developed. For example, mass spectrometry (MS) is being used in conjunction with HPLC to selectively detect N-nitrosamines in some matrices. The increased knowledge about the nature of nitrosamines will enable us to developing effective means of blocking their carcinogenic action and minimizing exposure. These advances will potentially lead to more definitive regulatory guidelines.

References

1. AOAC International, *AOAC Official Methods of Analysis*, 16th Edition, Method 987.05, 1995.
2. Edlefsen, Miriam and Brewer, M. Susan, "Nitrates/Nitrites." The National Food Safety Database, www.foodsafety.org/il/il089.html.
3. Food and Drug Administration, "Guidance for Industry: Submission of Documentation in Drug Applications for Container Closure Systems Used for the Packaging of Human Drug and Biologics." U.S. Department of Health and Human Services, June 1997.
4. Harsy, Stephen G., "The chemistry and analysis of N-nitrosamines." *Vanguard*, Corning Hazleton, Madison, WI, June 1995.
5. Loeppky, Richard N. and Michejda, Christopher J. (eds.), *Nitrosamines and Related N-Nitroso Compounds: Chemistry and Biochemistry*. American Chemical Society, Washington, DC, 1994.





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